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Quantitative Detection of Increasing HIV Type 1 Antibodies after Seroconversion: A Simple Assay for Detecting Recent HIV Infection and Estimating Incidence

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ABSTRACT

We have devised a simple enzyme immunoassay (EIA) that detects increasing levels of anti-HIV IgG after seroconversion and can be used for detecting recent HIV-1 infection. Use of a branched peptide that included gp41 immunodominant sequences from HIV-1 subtypes B, E, and D allowed similar detection of HIV-specific antibodies among various subtypes. Because of the competitive nature of the capture EIA, a gradual increase in the proportion of HIV-1-specific IgG in total IgG was observed for 2 years after seroconversion. This was in contrast to results obtained with the conventional EIA using the same antigen in solid phase, which plateaus soon after seroconversion. The assay was used to test 622 longitudinal specimens from 139 incident infections in the United States (subtype B) and in Thailand (subtypes B and E). The assay was also performed with an additional 8 M urea incubation step to assess the contribution of high-avidity antibodies. Normalized optical density (OD-n) was calculated ($OD_{specimen}/OD_{calibrator}$), using a calibrator specimen. An incremental analysis indicated that a cutoff of 1.0 OD-n and a seroconversion period of 160 days offered the best combination of sensitivity and specificity for classifying incident or long-term infections. The urea step increased the seroconversion period to 180 days with similar sensitivity and specificity. Separate analysis of B and E subtype specimens yielded the same optimal OD-n threshold and similar seroconversion periods. The assay was further validated in African specimens (subtypes A, C, and D) where the observed incidence was within 10% of the expected incidence. This assay should be useful for detecting recent HIV-1 infection and for estimating incidence among diverse HIV-1 subtypes worldwide.

INTRODUCTION

PREVALENCE AND INCIDENCE are measures of the extent and spread, respectively, of a disease in a population. Provided there is a convenient and reliable means of detecting disease, prevalence can be easily measured from a cross-sectional study of a population. Disease incidence, the proportion of people within a susceptible population who acquire the disease over a given period of time, is more difficult to measure and generally requires prospective follow-up in defined cohorts. The need for longitudinal follow-up could be circumvented if there were a means of distinguishing persons with recent versus long-term infection within the prevalent population.

Human immunodeficiency virus type 1 (HIV-1) infection is a case in point. Its natural history has features that evolve and could be used to distinguish recent from long-term infection.[1–7] Early infection comprises the period from exposure to seroconversion, during which HIV-1 antigen or nucleic acid may be detected, and the period after seroconversion, during which the antibody response evolves and matures. Testing strategies based on detecting the preseroconversion "window,"[8] although theoretically sound, have some practical drawbacks. Because

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the preseroconversion window is relatively short, large sample sizes are required to achieve reasonable confidence intervals for incidence estimates. This strategy would also require screening seronegative members of the population. A test applied to the seropositive population would be more economical and generally more practical. Evolving features of the antibody response that could be useful in distinguishing infection status include changes in antibody isotype, quantity, avidity, and specificity.[7]

A testing strategy involving a modified, "less sensitive" commercial HIV-1 antibody assay (3A11-LS) has been used for estimating seroincidence using cross-sectional specimens.[9] It is based on the differential titer of HIV-1 antibodies between recent and long-term infection. The assay is performed at a single dilution (1:20,000) and has been calibrated to define a threshold below which a sample is classified as recent infection. The proportion of infected persons found to be recently infected can then be used to estimate incidence. The assay performs well and has been used to estimate incidence in various U.S. populations. However, it has not been widely applied because of problems with availability (it relies on an early-generation commercial test), the requirement for dedicated equipment, and observations indicating performance differences when applied to populations infected with non-B HIV-1 subtypes.[10] Another readily available commercial enzyme immunoassay (EIA) has been modified to be less sensitive (OT-LS) for detection of recent infection. However, it also uses subtype B antigen and is based on the same principle (differential titer), making it likely to have variable subtype-specific performance. Development of a subtype-independent assay to detect recent infection and estimate incidence would be highly desirable for its worldwide application.

We have evaluated a number of approaches, based on different principles, for their ability to distinguish recent from established HIV-1 infection.[7] One such test, the anti-HIV-1 IgG capture assay, reflects the proportion of anti-HIV-1 IgG present in the serum and appears to perform particularly well. The test is performed in a single-well, EIA format and is suitable for high-throughput testing (96-well format). To overcome the problems associated with testing different subtypes, we have designed a branched synthetic peptide that incorporates immunodominant gp41 sequences form multiple subtypes. This peptide-based, capture EIA was used to measure the gp41 antibody content in sequential specimens after seroconversion. In parallel, we added and evaluated a urea elution step, as a surrogate for measuring avidity, so that the assay reflects both quantitative and qualitative aspects of the antibody response. Using well-characterized longitudinal specimens, collected in the United States and Thailand from subjects infected with subtype B or E, we determined the optimal conditions to calculate seroincidence. We present the application of this approach for predicting seroincidence in cross-sectional studies.

MATERIALS AND METHODS

Specimens

A total of 622 serum or plasma specimens from persons with known seroconversions (documented by serial testing) were used in this study: 67 specimens were from 22 commercially available seroconversion panels (Boston Biomedica [Boston, MA] and NABI [Miami, FL]); 518 specimens were from 90 seroconvertors from a longitudinal study of injecting drug users conducted by the Bangkok Metropolitan Administration (BMA) in Thailand (104 specimens from 18 subtype B infections and 414 specimens from 72 subtype E infections[10–12]); and 37 specimens were from known incident U.S. cases resulting from either accidental exposure or injecting drug use. The average interval between specimen collections during the first year of seroconversion was 60 days and the average period of follow-up was 371 days. In addition, 49 specimens collected from 18 seroconvertors (14 subtype A, 3 subtype D, and 1 subtype C) from Kenya were tested. Serum specimens from AIDS patients ($n = 456$) were tested to assess the possibility of false classification among people with declining immune status. These patients included 262 U.S. women from the HIV Epidemiology Research Study (HERS), 72 U.S. men, and 122 persons from Thailand (7 subtype B and 115 subtype E). We also tested specimens from 178 subjects whose date of seroconversion was unknown but who were known to have been seropositive for at least 1 year.

gp41 peptides and BED peptide

All peptides were synthesized on a model 431 automatic peptide synthesizer (Applied Biosystems, Foster City, CA), using 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry according to the manufacturer's protocol with slight modifications only for the branched peptide. First, an FMOC-Lys [1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl; Dde]-OH (Novabiochem, San Diego, CA) was incorporated at the point of branching. Second, a *t*-butyloxycarbonyl (Boc)-protected instead of an Fmoc-protected amino acid was used at the N terminus of each branch to prevent further coupling. Finally, the Dde protective group was removed manually, using 2% hydrazine in dimethylformamide, after each branch was completed so that the next branch could be assembled. All peptides were partially purified by reversed-phase high-performance chromatography (Bio-Rad, Hercules, CA). The branched peptide antigen (BED peptide) included immunodominant sequences from the gp41 region of subtypes B, E, and D, linked via lysine (Fig. 1). Biotin was added to the N terminus of the core structure before cleavage of the peptide from the resin. The peptide was stored as a dry powder dessicated at room temperature. A stock solution of peptide at 1 mg/ml in dimethyl sulfoxide was made and stored in aliquots at −20°C.

Conventional enzyme immunoassay

Peptides were coated onto Immulon II microwell plates (Dynatech, Chantilly, VA) at 0.5 μg/well by incubating 100 μl of peptide (5 μg/ml) in 10 mM bicarbonate buffer, pH 9.4, overnight 4°C. Wells were washed with 10 mM phosphate-buffered saline (PBS), pH 7.2, containing 0.05% Tween 20 and blocked with milk buffer (5% nonfat dry milk powder in PBS–0.3% Tween 20) for 1 hr at 37°C. Serial 4-fold dilutions of serum specimens (starting at 1:400) were prepared in milk buffer, 100 μl was added to wells, and the plates were incubated for 1 hr at 37°C. After four washes, goat anti-human IgG peroxidase conjugate (Bio-Rad), diluted 1:5000 in milk buffer, was added and the plates were incubated for 30 min at 37°C.

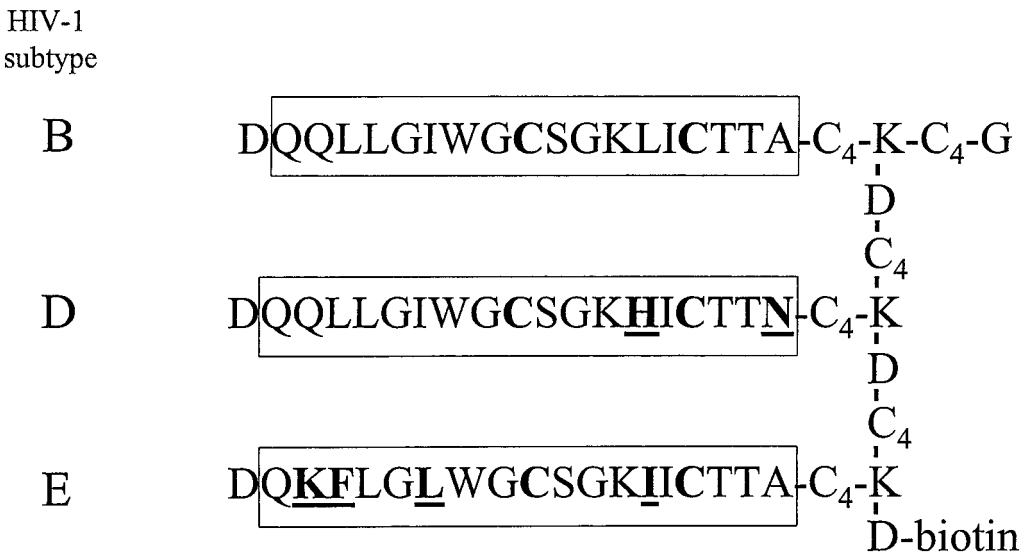

FIG. 1. Composition of the BED chimeric synthetic peptide derived from gp41 immunodominant sequences. C_4 represents four-aminobutyric acid spacers; aspartic acids (D) were added to increase solubility, and lysine (K) residues were used at the branching points. Cysteine residues are in boldface and differences in subtypes D and E sequences when compared with B sequences are underlined.

Conjugate was removed, wells were washed four times, and 100 μl of 2,2′,4,4′-tetramethyl benzidine (TMB) substrate (Kirkegaard & Perry, Gaithersburg, MD) was added. Color development was carried out at room temperature in the dark for 15 min and stopped by addition of 100 μl of 1 N H_2SO_4. Optical density (OD) was read at 450 nm with reference at 405 nm on a Dynex Thermo Labsystems, Helsinki, Finland, MRX spectrophotometer.

IgG-capture BED EIA

Wells of Immulon II plates were coated with 100 μl of goat anti-human IgG antibody (BioSource International, Camarillo, CA) at 2.5 g/ml in PBS, pH 7.2, by overnight incubation at 4°C. Wells were washed twice with 300 μl of wash buffer (0.1% Triton X-100 in PBS; pH 7.2) and then blocked by incubation with 300 μl of blocking/diluent buffer (3% bovine serum albumin, fraction V [Sigma, St. Louis, MO] in wash buffer) for 1 hr at 37°C. Wells were again washed twice with wash buffer. Sera (100 μl of 1:100 dilution in diluent buffer) were added to the plates, and the plates were incubated for 1 hr at 37°C. The wells were washed four times with 300 μl of wash buffer (plates were rotated 180° after two washes to minimize variations in washing steps). The BED–biotin peptide antigen (1 mg/ml in dimethyl sulfoxide) was diluted to 1 ng/ml in diluent buffer, 100 μl was added to each well, and the plates were incubated for 1 hr at 37°C. Wells were washed again four times as described above, and 100 μl of streptavidin–peroxidase diluted 1:8000 in diluent buffer [BioSource International] was added to each well. Enzyme conjugate was incubated for 90 min at 37°C, followed by four washes. The TMB substrate (100 μl) was added to each well, and the plates were incubated for 15 min at 25°C (incubator). Color development was stopped by addition of 100 μl of 1 N H_2SO_4. The OD values were read at 450 nm with 405 nm as reference wavelength, using a Dynex MRX spectrophotometer. All specimens were tested in duplicate. Appropriate negative (NC) and positive (PC) control sera were included in triplicate on every plate. A PC specimen at a predetermined dilution of 1:20 in the NC was also included on every plate (in triplicate) as a "calibrator" to be used for normalizing optical density values to minimize the interrun variations. The OD values were normalized as follows: normalized OD (OD-n) = mean specimen OD/mean calibrator OD. This calculation resulted in a lower interrun variation than did subtraction of the NC from either the numerator (as in the 3A11-LS assay) or from both the numerator and denominator. All reagent concentrations were selected to be optimal based on pretitration studies.

IgG-capture BED–urea EIA

The above-described protocol was modified by the addition of a step after incubation with BED–biotin peptide. BED–biotin peptide bound to low-avidity antibodies[13] was dissociated by adding 100 μl of 8 M urea and incubating for 15 min at 37°C. Controls were treated in the same manner. Urea was removed, and wells were washed four times with wash buffer. Subsequent conjugate and substrate incubation steps were the same as in the above-described protocol. As shown previously, 8 M urea dissociates the BED peptide from low-avidity anti-HIV IgG but does not affect the capture of human IgG or the binding of the anti-human IgG antibodies to the plate.[7]

Data analysis

Days since seroconversion were calculated by assuming that the date of seroconversion was the midpoint between the last negative and first positive HIV antibody test results. OD-n values were plotted against days since seroconversion. A specimen was defined as seroincident if its OD-n value fell below a

certain threshold (cutoff). Specimens registering below this threshold, in turn, were defined as having seroconverted within a designated period of time (seroconversion interval). Optimal threshold cutoff (OD-n) and seroconversion interval were determined by calculating sensitivity and specificity of all combinations of cutoff values between 0.5 to 1.5 OD-n (in increments of 0.1) and seroconversion intervals of 100 to 200 days (in increments of 10 days). For IgG-capture BED–urea EIA, the duration examined was from 120 to 220 days.

Given the optimal cutoff for OD-n from the preceding analysis, a statistical modeling approach was used to define the seroconversion interval represented by this cutoff. For each individual, data were linearized by taking the square root of OD-n and the log of time. A linear mixed-effect model was applied to estimate the slope and intercept for each person, while using information from the entire sample to bias the estimates toward the mean.[14] The seroconversion intervals were determined as the time from when the regression lines intercepted baseline to the time they intercepted the cutoff OD-n. Baseline OD-n was assigned for each regression from a random selection of ODs that matched the distribution (mean ± SD) of antibody-negative sera. To estimate variability in the seroconversion intervals, 1000 bootstrap estimates were calculated by randomly adding the error deviations from the original regression to the ODs, recalculating the linear mixed-effect model, and then computing a new set of intervals.[15] Confidence intervals for the mean were calculated using the bias-corrected and accelerated method described by Efron and Tibshirani.[16]

RESULTS

Our previous results, using the less-sensitive 3A11-LS assay, demonstrated that the 3A11-LS assay has different performance characteristics among individuals infected with HIV-1 subtype B or E.[10] The use of subtype B viral lysate as antigen in this assay appeared to contribute to these differences. To confirm this hypothesis, serum specimens taken at about 1 year after seroconversion from 12 individuals (6 subtype B and 6 subtype E) were titered on plates coated with gp41-B peptide, gp41-E peptide, or gp41-B and p41-E peptides. Titers were significantly higher with homologous peptides and lower with heterologous peptides (Fig. 2, left and middle). When the plates were coated with both peptides together, equivalent end-point titers were obtained among individuals infected with subtype B or E in Thailand (Fig. 2, right). These data strongly suggested that antigens derived from multiple subtypes should be included for equivalent detection of quantitative humoral responses across subtypes.

We synthesized a branched-chain peptide (BED peptide) that included consensus immunodominant gp41 sequences from subtypes B, E, and D (Fig. 1, see Materials and Methods). A close examination of homologous sequences derived from various HIV-1 subtypes suggested that this sequence was well conserved and that inclusion of sequences from the three subtypes would be sufficient to cover major HIV-1 subtypes prevalent in different areas of the world. The BED peptide was universally reactive with sera from infected people. All sera in our collection that were confirmed positive by conventional serologic testing were reactive with the BED peptide.

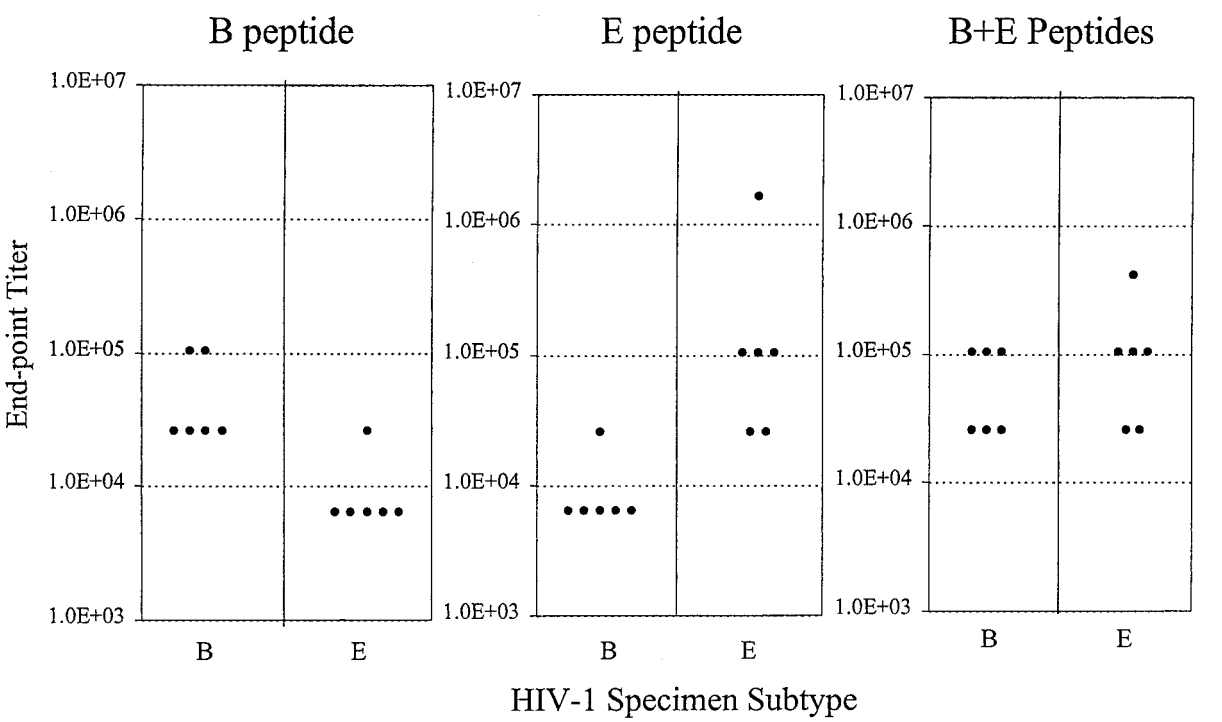

FIG. 2. Antibody titers to gp41 peptides derived from subtype B, subtype E, or subtypes B and E together. Specimens collected about 1 year postseroconversion from 12 seroconvertors (6 subtype B and 6 subtype E) were tested.

To demonstrate the differences between the conventional EIA (using BED peptide-coated plates) and the IgG-capture BED EIA, longitudinal specimens from Thai seroconvertors were tested by the two assays. The OD values from two representative individuals were plotted against the days since seroconversion (Fig. 3). The results show that OD values obtained by conventional EIA plateau soon after seroconversion (1:400 dilution). In contrast, the IgG-capture BED EIA showed a gradual increase in OD values (1:100 dilution) for almost 2 years after the estimated time of seroconversion, indicating an increase in the proportion of HIV IgG present in the sera. This approach also had a better dynamic range than the direct EIA done at a higher dilution (e.g., 1:20,000, as is done with the 3A11-LS assay).

We used the IgG-capture BED EIA, with or without the urea dissociation step, to test specimens from seroconverting individuals. The distribution of OD-n with respect to time since seroconversion for 622 longitudinal specimens is shown in Fig. 4. These data include subtype B and subtype E incident infections from the United States and Thailand. Figure 4A shows the results of the IgG-capture BED EIA, whereas Fig. 4B shows the results of the IgG-capture BED–urea EIA, with an additional 8 M urea incubation step. Both assays showed a gradual increase in the proportion of HIV-specific antibodies with time after seroconversion. The distribution of OD-n fell within a narrow range early after seroconversion but broadened over time. The horizontal and vertical lines represent the optimal cutoff value (OD-n) and optimal seroconversion interval, respectively, based on the sensitivity and specificity optima described below. These demarcations define "recent infections" as true positives (lower left quadrant) and "long-term infections" as true negatives (upper right quadrant) as well as false negatives (upper left quadrant) and false positives (lower right quadrant).

This assay was devised to discriminate early from later infection. This requires selection of an OD-n below which a specimen is defined as incident (optimum cutoff value) and definition of the duration of seroconversion that the selected OD-n represents. The selection of assay thresholds is arbitrary but should be based on a rationale that results in optimal performance of the assay. We initially analyzed our data empirically and then refined and affirmed the selection of the assay thresholds by a statistical modeling approach. In the empirical approach, both cutoffs were changed incrementally, and the proportion of all data that fell below both cutoffs (true positives, lower left quadrant of Fig. 4) and above both cutoffs (true negatives, upper right quadrant of Fig. 4) was determined for all combinations of cutoffs. The optimal cutoffs were an OD-n of 1.0 and a seroconversion duration of 160 days for the IgG-capture BED EIA versus 180 days for the IgG-capture BED–urea EIA (Fig. 5), corresponding to the peak on the surface curves, respectively. For the IgG-capture BED EIA cutoffs, sensitivity was 81.7%, specificity was 89.1%, and accuracy in the estimate of incidence was 93% (due to compensatory effects of false positives and false negatives) (Fig. 4 and Table 1). For the IgG-capture urea–BED EIA, similar sensitivity (88.2%), specificity (82.3%), and accuracy (93%) were obtained. Because urea

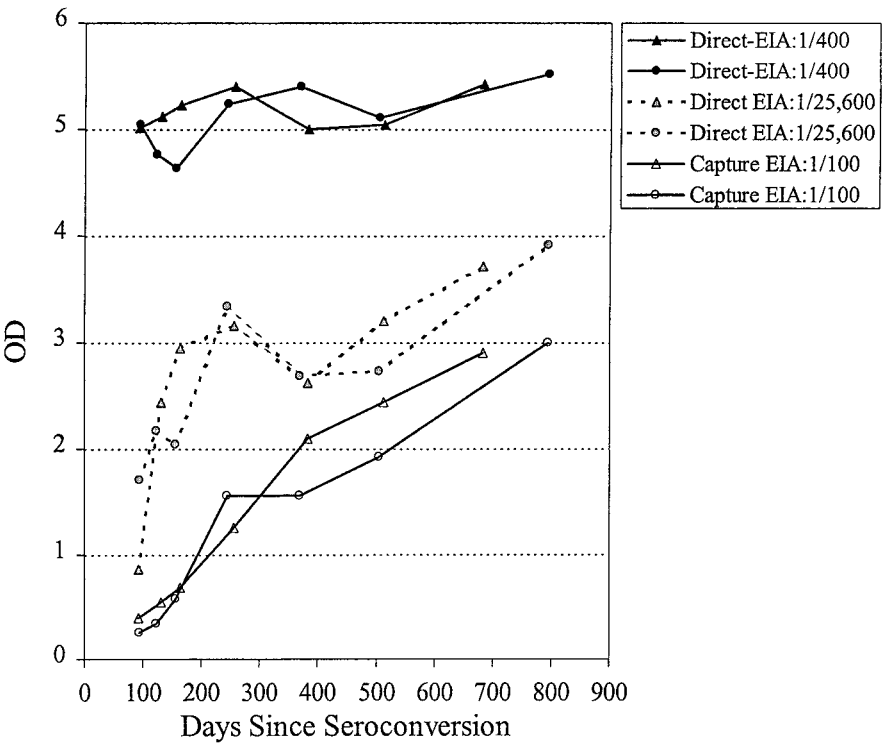

FIG. 3. Optical density (OD) values obtained when longitudinal specimens from two seroconvertors were assayed by a conventional solid-phase BED EIA at 1:400 (●, ▲) and 1:25,600 (●, ▲) dilutions or by IgG-capture BED EIA (○, △). The same gp41 BED peptide was used in both assays. Circles (solid, gray, and open) and triangles (solid, gray, and open) represent the two individuals.

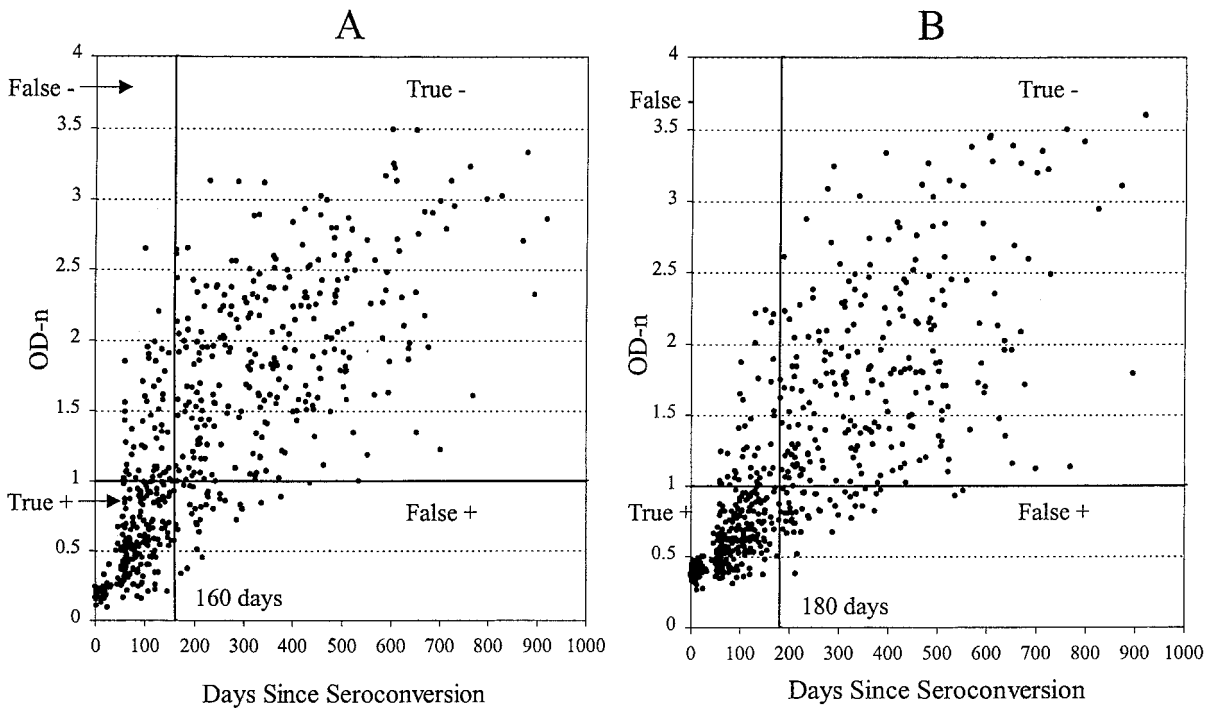

FIG. 4. Normalized OD (OD-n) distribution for all subtype B and E specimens ($n = 622$) with respect to their estimated days since seroconversion. (**A**) OD-n by IgG-capture BED EIA; (**B**) OD-n by IgG-capture BED–urea EIA. The solid horizontal line represents the threshold cutoff of 1.0; the vertical line represents the optimal seroconversion duration of 160 days (**A**) or 180 days (**B**) for detecting recent infection.

treatment involves one more step without any additional advantage, further analysis was limited to the IgG-capture BED EIA.

Given an OD-n cutoff of 1.0, we performed an additional statistical analysis to determine the seroconversion duration that such an OD-n represents. In a linear mixed-effects model in which seroconversion duration was estimated from the slope and intercept of linearized regression plots for each person, a seroconversion duration of 158 days (95% CI, 151–163 days) was obtained, compared with 160 days for the empirical estimate.

The same analyses were applied separately to the Thai B and E specimens (Fig. 6). In the empirical analysis, optimal classification was achieved at an OD-n cutoff of 1.0 and seroconversion duration of 170 days for subtype B and 150 days for subtype E, compared with 160 days for the combined data. In the statistical projection (Fig. 7), the seroconversion duration at a cutoff was 1.0 was also somewhat longer for subtype B compared with subtype E: 186 days for subtype B, 152 days for subtype E, and 158 days for the combined data.

Although the 3A11-LS assay performed differently with subtype B and E specimens, it performed well with subtype B specimens (for which the assay was originally calibrated).[10] We compared the results of the 3A11-LS and IgG-capture BED EIA for subtype B specimens only (Fig. 8). There was good agreement between the two assays, with a correlation coefficient (r) of 0.90. Of 104 specimens, 42 (40.4%) and 47 (45.2%) were classified as incident sera by the 3A11-LS and IgG-capture BED EIA, respectively. Overall, there was agreement for 91 (87.5%) of 104 specimens in classification of subtype B specimens.

We further examined assay performance by comparing the observed incidence with the expected incidence in defined subsets of specimens (Table 1). All Thai and U.S. specimens were used for assay calibration, and the observed incidence was 92.6% of the expected value. When separately analyzed, Thai subtype E, Thai subtype B, and U.S. subtype B all gave percent seroincidence values close to what was expected. Further validation was achieved by applying the assay to a cohort of longitudinal specimens from Kenya with subtype A, D, and C incident infections (Table 1). These Kenyan specimens were not used for determination of the assay parameters and calibration. The estimated incidence among these specimens was 107% of the actual value.

The accuracy of the assay was affected by the magnitude of uncertainty in the timing of seroconversion (i.e., interval between last negative [LN] and first positive [FP] specimen). For statistical purposes, we chose the midpoint of the interval as the seroconversion date. This results in an uncertainty of (FP − LN)/2 days for individual serum specimens. When groups of specimens with varying mean uncertainty were examined separately, those with the lowest mean uncertainty (10 days, $n = 50$) were all (100%) correctly classified; this accuracy declined gradually to 73% with increasing uncertainty of 140 days.

Sera from AIDS patients (United States and Thailand) were also tested by the IgG-capture BED EIA to assess the potential

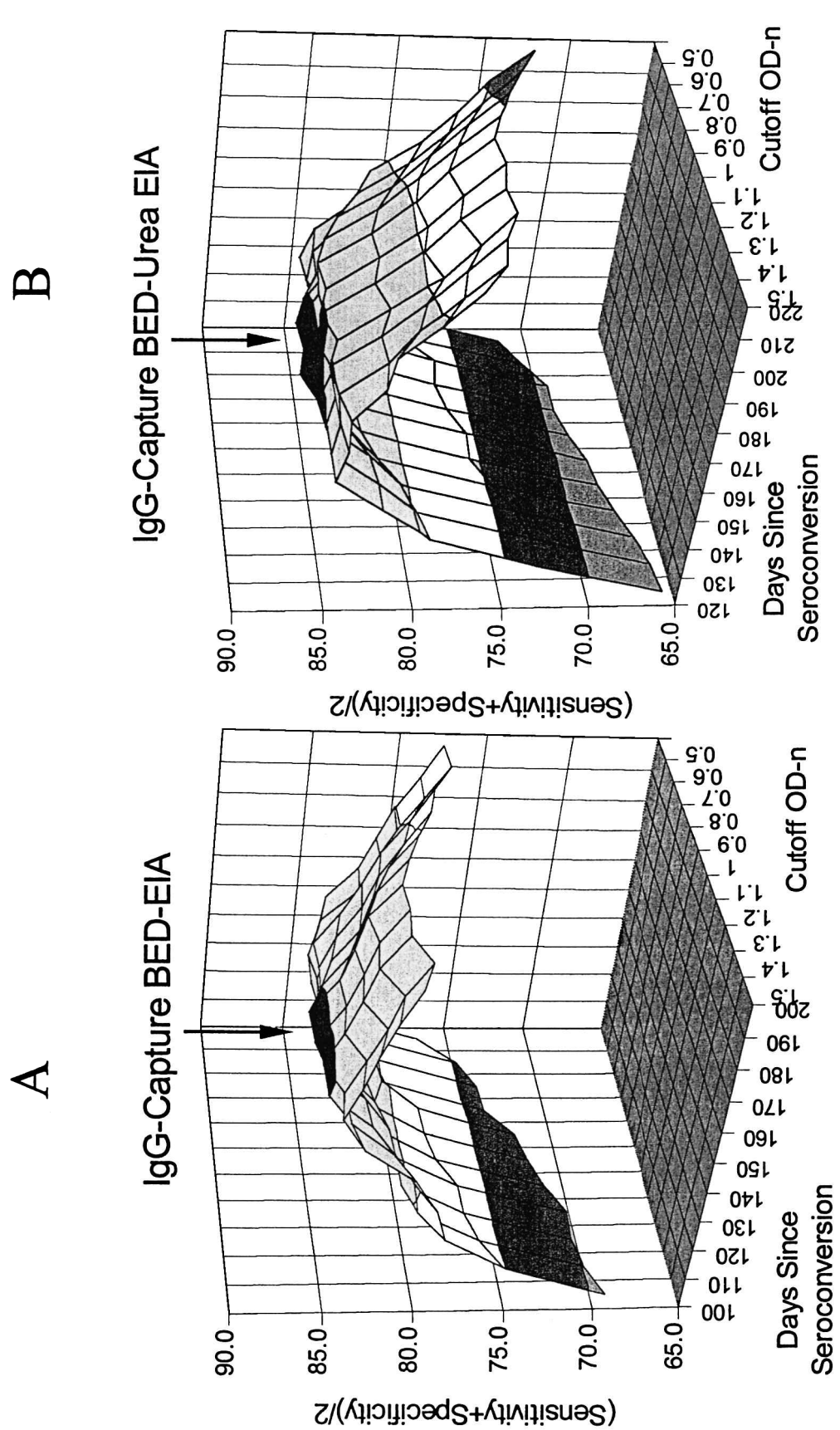

FIG. 5. Three-dimensional surface plots showing sensitivity and specificity [(sensitivity + specificity)/2] at various combinations of OD-n cutoff and time since seroconversion by IgG-capture BED EIA (**A**) and IgG-capture BED–urea EIA (**B**). Arrows indicate optimal combination for each of the assays.

TABLE 1. VALIDATION OF ASSAY PARAMETERS IN INDIVIDUAL COHORTS AND AN ADDITIONAL KENYAN COHORT NOT USED FOR CALIBRATION

Population	n[a]	*Observed (%) incident*	*Expected (%) incident*	*Accuracy*[b] *(%)*
Thai				
Subtype E[c]	414	37.4	42.0	89.1
Subtype B[c]	104	45.2	45.2	100.0
U.S.A., subtype B[c]	104	82.7	86.5	95.6
All Thai and U.S.A.[c]	622	46.3	50.0	92.6
Kenya[d] (A, D, and C)	49	57.1	53.1	107.7

[a]Total number of specimens tested.
[b]Accuracy (%) = (observed seroincident/expected seroincident) × 100.
[c]Specimens used to define the assay parameters.
[d]These specimens were not used to define the assay parameters.

for false classification of individuals with declining antibodies as recent infections. Of 456 specimens from AIDS patients, 20 or 4.38% registered below the threshold cutoff of 1.0 and would have been misclassified as recent infection. We also tested 178 specimens from HIV-1-infected people without AIDS who were known to be infected for more than 1 year (although the date of seroconversion was not known). Of these 178 specimens, 3 or 1.69% registered as recent infections.

DISCUSSION

Application of a less sensitive EIA (3A11-LS) to distinguish recently infected people from those with established infection was a simple yet important development,[9] allowing measurement of incidence using cross-sectional specimens from a population. However, this assay is a 60-well, bead-format EIA, requires dedicated equipment from the manufacturer, and is not

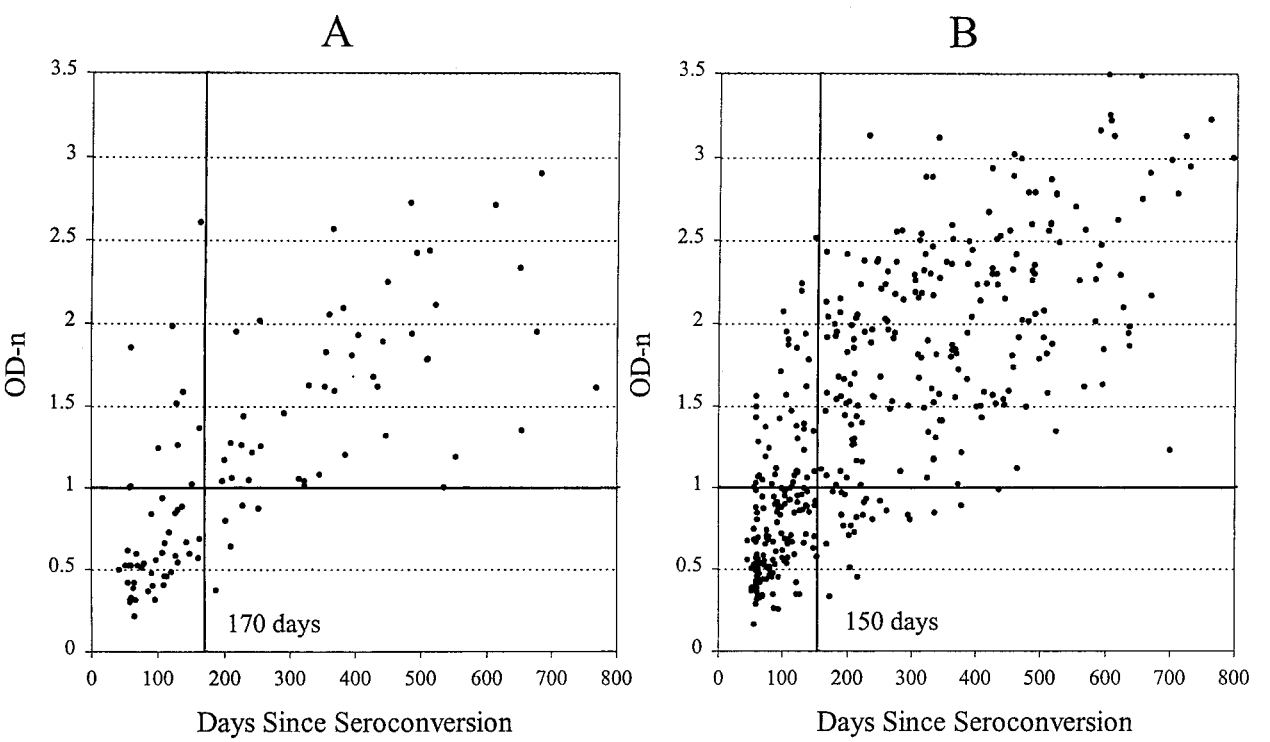

FIG. 6. Normalized OD (OD-n) distribution for Thai B specimens (n = 104) from 18 seroconvertors (**A**) and for Thai E specimens (n = 414) from 72 seroconvertors (**B**) with respect to their estimated days since seroconversion. The solid horizontal lines represent the threshold cutoff of 1.0; the vertical lines represent the optimal seroconversion duration of 170 days (subtype B) or 150 days (subtype E) to achieve optimal classification.

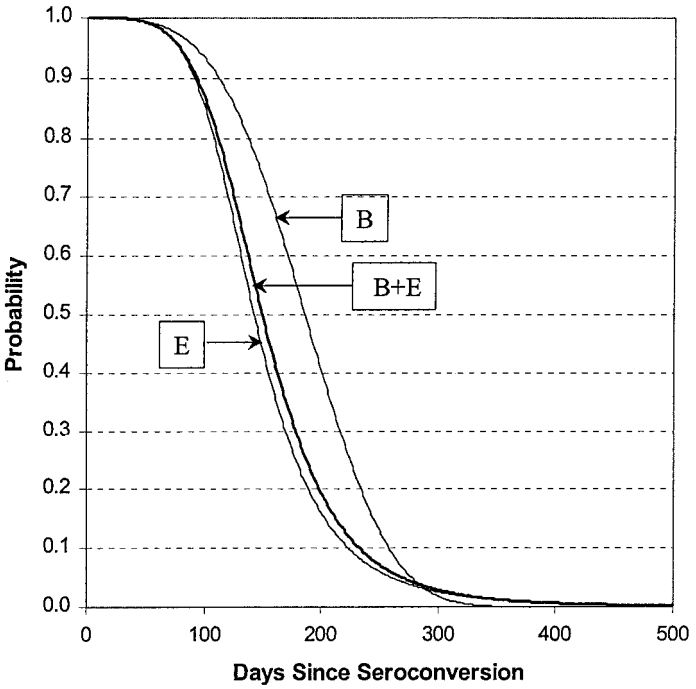

FIG. 7. Mixed-effects modeling approach showing the probability that specimens will register an OD-n value of less than 1.0 as a function of time since seroconversion. Analyses for Thai subtypes B, E, and B+E are shown. Extrapolation at a probability of 0.5 determines the mean time since seroconversion for each population.

widely available. In addition, our more recent work indicated that the assay performed differently among different HIV-1 subtypes,[10] precluding its useful application in many parts of the world with divergent, multiple subtypes. Addition of the OT-LS assay to detect recent infection has alleviated some of the problems (availability, 96-well format). However, it still requires a 1:20,000 dilution of specimens, which is labor intensive, less precise, and may contribute to high variability. Moreover, it is likely to perform differently with different subtypes (uses only subtype B antigens). The IgG-capture BED EIA described here was developed to address some of these shortcomings. Incorporation of a branched gp41 antigen derived from multiple subtypes allows equivalent detection of antibody levels regardless of subtype. Moreover, the capture format of the EIA allows the assay to be performed at a 1:100 dilution of specimen, compared with the 1:20,000 dilution required by the less-sensitive assays. In a direct comparison, the IgG-capture BED EIA performed well across subtypes (Table 1), whereas parallel testing of specimens from Thailand[10] and Ivory Coast (data not shown) with the 3A11-LS assay markedly overestimated the incidence of subtype E and A, respectively.

Antibodies to the gp41 immunodominant region (gp41-IDR) appear early and are important for diagnostic assays. Indeed, assays have been designed to use exclusively peptide antigens derived from this region. Anti-gp41-IDR antibodies also play a major role in assays that use viral lysate or recombinant or peptide antigens. Fine mapping of this epitope with human sera has identified critical amino acid residues for optimal antibody binding.[17] The gp41-IDR is conserved, and a single peptide is adequate to detect antibodies to different HIV-1 subtypes. However, we have shown that antibody titers to gp41-IDR are higher against a homologous peptide than to a heterologous peptide even 1 year after seroconversion (Fig. 2). Thus, although it is possible to detect HIV-1 antibodies qualitatively, using a single gp41-IDR peptide, quantitative detection of antibodies during seroconversion requires antigens derived from multiple sequences to achieve equivalent results. To address this problem, we designed a branched gp41-IDR peptide (BED peptide; Fig. 1). Consensus sequences from subtypes B, E, and D were found to be sufficiently divergent and representative of the major (M) subtypes of HIV-1. Designing a single branched peptide ensured that each subtype was present in equimolar amounts. Connecting the peptides by four aminobutyric acid spacers (C_4) allowed greater accessibility and free rotation for optimal antibody binding. The BED peptide was reactive with all anti-HIV sera in our collection (subtypes A–E) and appeared to be equivalently reactive among subtypes as assessed by saturation binding and end-point titers (data not shown). In the capture format described here, the observed seroconversion periods for subtypes B and E were 170 and 150 days, respectively. This small difference could be due to a slower HIV IgG synthesis in subtype B infection than in subtype E infection or to a slightly less efficient recognition of the subtype B peptide by homologous antibodies. A study using specimens from the same Thai cohort reported that individuals infected with subtype E have significantly higher viral RNA levels compared with those with subtype B infections in the first 3 months of infection.[11] Lower viral load in subtype B infection resulting in slower kinetics of HIV-specific IgG synthesis could explain the observed differences in the seroconversion interval. Alternatively, the possi-

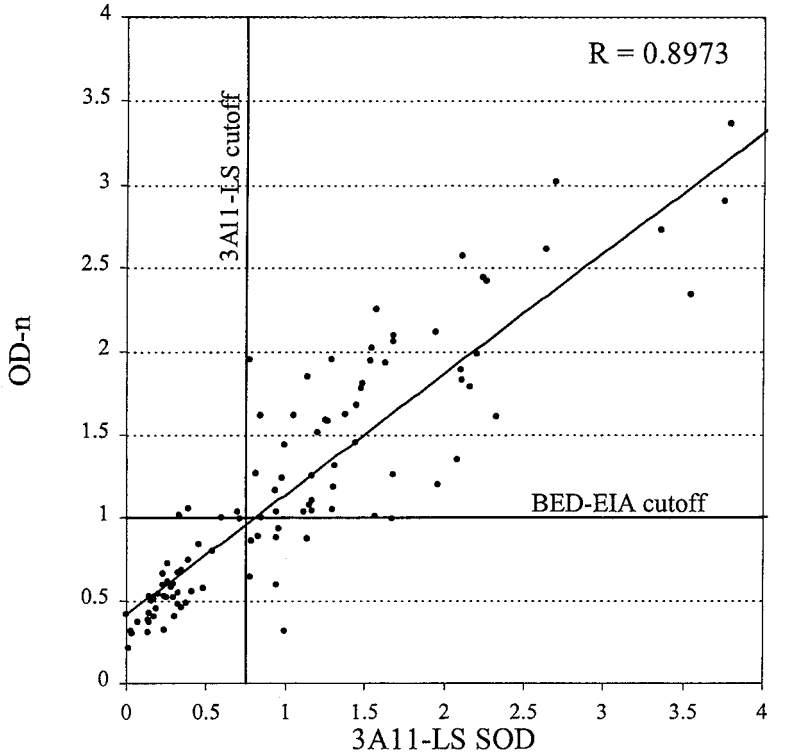

FIG. 8. Correlation between 3A11-LS standardized OD (SOD) and IgG-capture BED EIA OD-n for subtype B seroconvertors from Thailand. Linear regression line is shown with *r* value.

bility of somewhat poor recognition of the B peptide by B sera is also likely as heterogeneity of subtype B (9–10%) is greater than that of subtype E (5–6%) in Thailand.[12]

In addition to similar reactivity across subtypes, the IgG-capture BED EIA, because of its competitive nature, has several important features that make it especially useful for detecting recent infections in population studies. First, the assay has a useful dynamic range: that is, the assay registers incremental OD-n values over a broad range of HIV antibody content and does so in a single-well format (Figs. 3, 4, and 6). Because the IgG-capture BED EIA results reflect the proportion of HIV IgG present in the serum, the final dilution or the precision of the serum dilution is not important as long as IgG in the serum is in excess. Indeed, all the reagents in the assay are present in excess or saturating amounts, which results in excellent reproducibility. Certain technical features of the assay also improve reproducibility. Expression of results by reference to a calibrator as a simple ratio (specimen OD/calibrator OD) reduced interrun variation compared with that of OD alone. Subtraction of the negative control specimen OD actually increased the coefficient of variation (CV) and was not done. Further, an OD-n of 1.0, selected as the optimal cutoff, is in the range of the assay where the CV is minimal (5–10%). In our attempts to further refine the assay, we added a urea incubation step to the IgG-capture BED EIA to dissociate low-avidity antibodies. This step increased the seroconversion duration, but sensitivity and specificity remained at about 85%, thus offering no additional advantage (Figs. 4 and 5). Given the inherent variability of the immune response, it is likely that the 85% sensitivity and specificity observed here may be close to the limits that would be expected from any serologic approach to the detection of recent infection.

Our results demonstrate that the IgG-capture BED EIA offers a simple and promising approach to detect recent HIV-1 infection and estimate incidence among persons infected with diverse HIV-1 subtypes worldwide. This assay should have wide applications for monitoring the epidemic, developing and targeting prevention programs, selecting cohorts for vaccine trials, assessing the impact of community interventions, and monitoring for spread of subtype variants and drug resistance among new infections in populations.

APPENDIX

Application of the IgG-capture BED EIA to the estimation of incidence

By virtue of its capacity to distinguish recent from established infection, this assay was devised to estimate incidence in a population by cross-sectional sampling, screening for seropositives, and testing the seropositives for recent infection. If the number of seropositives that register as recently infected (within 160 days since seroconversion) is N_i, the yearly number is given by $F_1 N_i$ where F_1 is a correction factor for the seroconversion interval (F_1 = 365 days/160 days = 2.28). This assumes that the rate of new infections has been steady over the past year (or will be steady in the ensuing year). The yearly

incidence observed (I_o) in the susceptible population is then calculated from the formula

$$I_o = \frac{F_1 N_i}{N_{sn} + F_1 N_i} \times 100$$

where N_{sn} is the number of seronegatives in the population.

A less obvious correction relates to the nature of sensitivity/specificity analysis that is derived from a data set heavily weighted toward early infection and that makes no adjustment for misclassification.[18] Figure 9A shows a plot of percent positivity as a function of infection duration. This was obtained by calculating for each day the mean percent positivity for a period of 15 days before and 15 days after each day. A smoothing function was applied to these data and the curve is asymptotic to a minimum percent positivity of 1.69% (the percent positivity in people infected for more than 1 year). From an integration of this plot, a net sensitivity of 82.7% and specificity

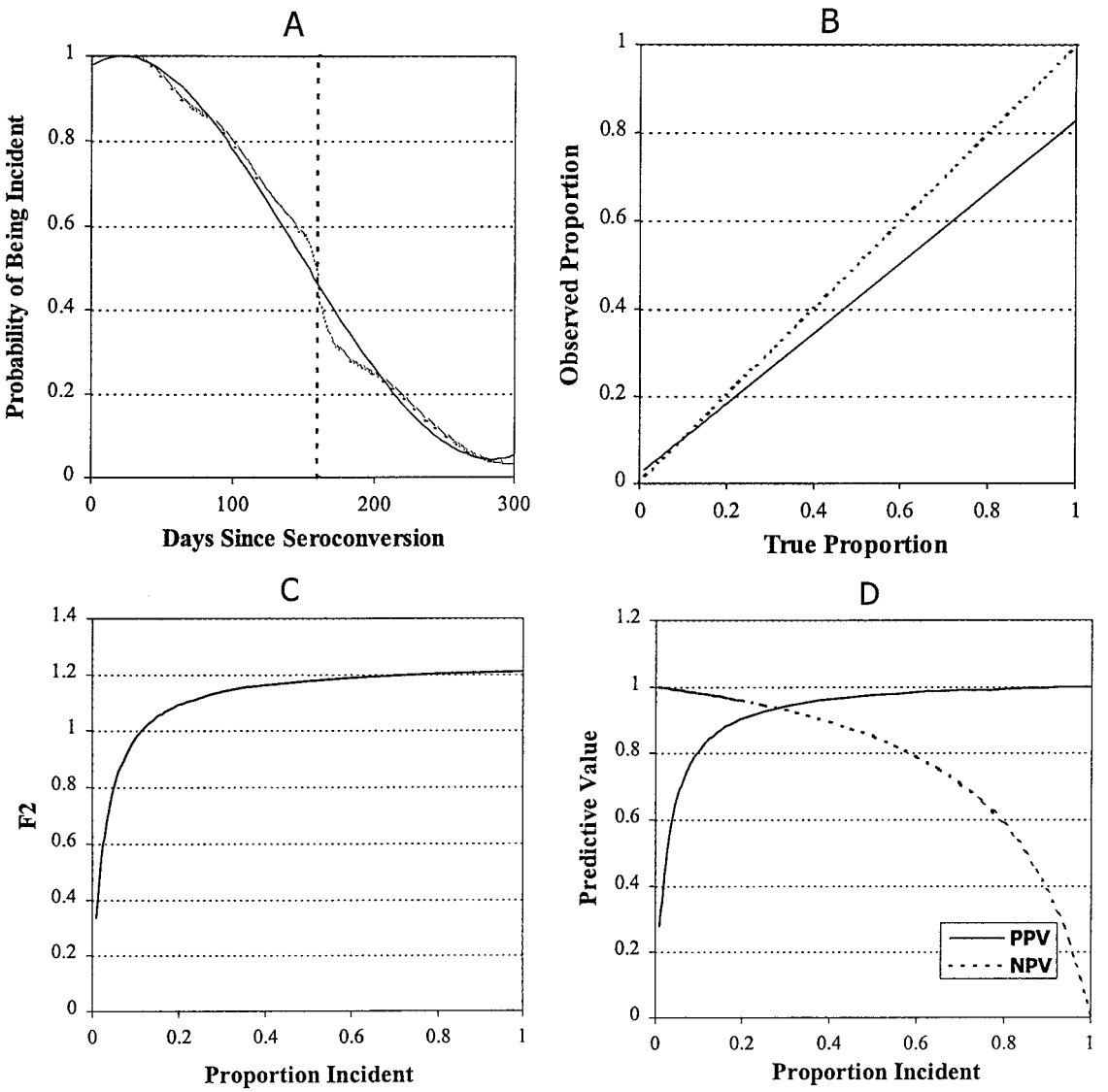

FIG. 9. Application of the BED-IgG capture assay to population studies. (**A**) Proportion of specimens registering an OD-n of <1.0 (seroincident positive) as a function of time since seroconversion. Polynomial best fit of data is shown as a solid line. The net percent positive (OD-n < 1.0) for the period 0 to 160 days (demarcated by the vertical line) is 82.7% (sensitivity) and for the period of 161 days to 10 years is 2.2% (100 − specificity). (**B**) Relationship of the true proportion to the observed proportion of seroincident specimens in the seropositive population, given a sensitivity of 82.7% and specificity of 97.8% (—). Diagonal dotted line (– – –) is the plot when the true and observed proportions are the same. (**C**) Plot of F_2 versus observed proportion of seroincident specimens in the seropositive population. F_2 is the correction factor by which the observed number of positive test results must be multiplied to obtain the true number. The plot is for a test having 82.7% sensitivity and 97.8% specificity. (**D**) Positive predictive value (—) (proportion of positive test results that are true positives) and negative predictive value (– – –) (proportion of negative test results that are true negatives) as a function of the proportion of test specimens that are positive given a sensitivity of 82.7% and specificity of 97.8%.

of 97.8% were obtained, assuming a random distribution of sampling for the period 0 days to 10 years.[19]

To some extent, false negatives and false positives may counterbalance each other in rendering an accurate determination of the number of seroincident specimens in the seroprevalent population (Table 1). The number of false positives and false negatives in a test population is a function of sensitivity, specificity, and the proportion of tested specimens that are truly seroincident. If sensitivity, specificity, and the observed proportion of seropositive specimens that test positive for seroincidence are known, the true proportion that are seroincident can be calculated. The relationship of the true proportion of incident specimens to the observed proportion of incident specimens (using sensitivity and specificity values given above) is displayed graphically in Fig. 9B. At low proportions, the observed proportion overestimates the true proportion and vice versa at higher proportions. To correct for the error in the estimate, we introduce the correction factor F_2 (true proportion/observed proportion) in terms of experimentally derived variables:

$$F_2 = \frac{P_{obs} + [\text{specificity}] - 1}{P_{obs}([\text{sensitivity}] + [\text{specificity}] - 1)}$$

where sensitivity and specificity are expressed as proportions and P_{obs} is the proportion of seropositive specimens that test seroincident-positive. If this factor is used to determine a corrected estimate of incidence (I_c), the formula is

$$I_c = \frac{F_1 F_2 N_i}{N_{sn} + F_1 F_2 N_i} \times 100$$

Figure 9C shows a plot of F_2 values as a function of a range of observed proportions, given a sensitivity of 82.7% and specificity of 97.8%.

The correction factor F_2 serves to improve the accuracy of the estimate but it relies on accurate measurement of sensitivity and specificity. Specificity, in particular, may not be reliably known in all populations. For instance, the false-positive rate appears to be somewhat higher in AIDS patients than in patients without AIDS who have been infected for longer than 1 year (4.38 vs. 1.69%). Inclusion of AIDS patients in the serosurvey for HIV positives would falsely elevate the estimate. However, if they compose less than 5–10% of the seropositives, the effect would be minimal.[9] These considerations may apply to other non-AIDS-defining conditions or infections as well. Thus, whereas application of this assay may be useful for cross-sectional estimates of incidence, it does not mitigate the need for careful selection of cohorts and examination for bias when comparing different populations.

Also shown in Fig. 9 are plots of the expected positive and negative predictive values. These are also functions of sensitivity, specificity, and the proportion of seropositive specimens that are truly incident. This test was envisioned and optimized for population studies. Because of potential false-positive and false-negative results, we would urge caution in the use of this test for diagnosis of recent infection in individual specimens. This underscores the general problem of using a reasonably specific test for diagnosis when pretest probability is low. Of course, a more stringent cutoff could be invoked to improve positive predictive value at the expense of negative predictive value and vice versa. For instance, invoking an OD-n cutoff of 0.5 and seroconversion interval of 1 year and applying these cutoffs to our data set resulted in a sensitivity of 28.2%, specificity of 100%, a positive predictive value of 100%, and a negative predictive value of 28.2%.

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